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Clonal Analysis of Hematopoietic Cells Using a Novel Polymorphic Site of the X Chromosome

Takahiro Okamoto,[1]* Masaya Okada,[1] Hiroshi Wada,[1] Akihisa Kanamaru,[2] Eizo Kakishita,[1] Tomoko Hashimoto,[3] and Junichi Furuyama[3]

[1]Second Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan
[2]Third Department of Internal Medicine, Kinki University School of Medicine, Japan
[3]Department of Genetics, Hyogo College of Medicine, Hyogo, Japan

Clonality of hematopoietic cells on a smale scale (nanogram amounts of DNA) can be detected by X-chromosome inactivation using the polymerase chain reaction (PCR). The human androgen-receptor gene (HUMARA) has a polymorphic short tandem repeat (STR), and has generally been used for clonality analysis since heterozygosity for the gene occurs in 90% of caucasian females. We examined heterozygosity of the STR on HUMARA in 110 Japanese females and found heterozygosity in 74 of 110 (67%). To examine for hematologic clonality in females with HUMARA homozygosity, we used a primer specific for a novel polymorphic STR site between DXS15 and DXS134 (DXS15-134) on Xq28. Heterozygosity for this site was found in 50 of 110 females (46%). Clonality of the hematopoietic cells was detected in 91 of 110 females (83%) using PCR of either the STR sites on HUMARA or DXS15-134. The X-inactivation patterns using PCR of DXS15-134 corresponded exactly with those obtained using PCR of HUMARA in 18 females who were heterozygous for both DXS15-134 and HUMARA. Using PCR of DXS15-134, we examined the clonality of bone marrow cells separated by flow cytometry in a patient with erythroleukemia (M6). Clonality was found not only in myeloid lineage cells but also in B lymphocytes. The clonality assay for DXS15-134 may be useful to assess for clonality of hematopoietic cells in the Japanese population, when combined with the HUMARA assay. Am. J. Hematol. 58:263–266, 1998.

Key words: clonality; polymerase chain reaction; short tandem repeat; polymorphism, X-inactivation

INTRODUCTION

The clonal origin of hematopoietic cells has been assessed by X-inactivation/methylation analysis in female patients heterozygous for X-linked DNA polymorphisms of hypoxanthine phosphoribosyl transferase (HPRT) [1,2], phosphoglycerate kinase (PGK) [3,4], and the variable number of tandem repeats at DXS225 (M27 β) [5] using the Southern blotting method. One of the major limitations of Southern blotting is the 5 to 20 μg of DNA required for the double enzyme digestions in clonality studies. In contrast, polymerase chain reaction (PCR) methods require as little as 1 ng of DNA from hematopoietic cells for clonal analysis. Thus, the PCR method makes it possible to analyze the clonality of cells from hematopoietic colonies grown in culture or after sorting of pure cell populations from patients with low frequencies of circulating cells. Polymorphic short tandem repeat (STR) sites of PGK and the human androgen-receptor gene (HUMARA) have been used for this PCR assay. The PCR method using PGK requires further cutting by the restriction enzyme *Bst*XI. Furthermore, the frequency of heterozygosity of STR on PGK is relatively low, around 30% [6]. HUMARA is generally used for clonality analysis [7,8], because the heterozygosity of STR on HUMARA approaches 90% in caucasian females [9]. Although the frequency of heterozygosity for HUMARA may be lower in the Japanese population, there are no reports about the heterozygote frequency of HUMARA

*Correspondence to: Takahiro Okamoto, M.D., Second Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo, 663 Japan.

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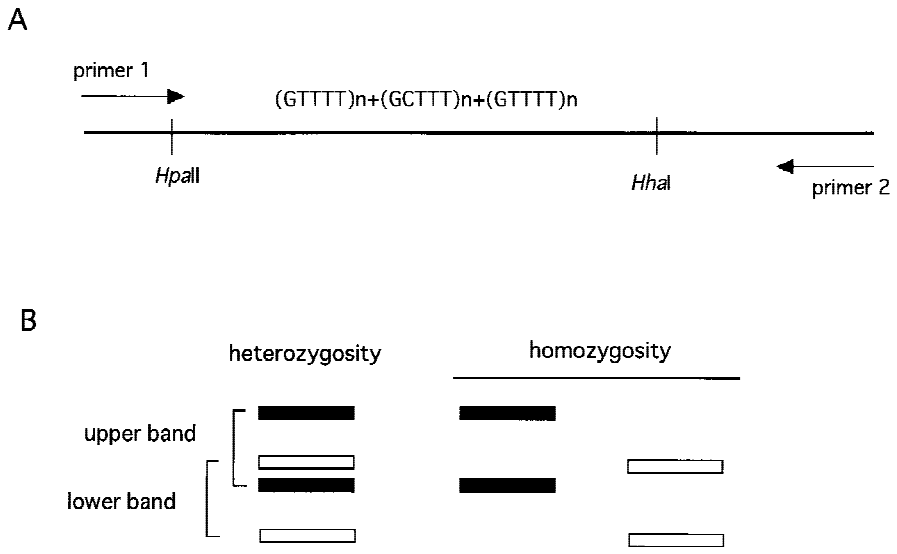

Fig. 1. A: Diagram of the region amplified between DXS15 and DXS134 (DXS15-134) on Xq28. One *Hpa*II and one *Hha*I site are located near the polymorphic STR. B: PCR products of DXS15-134 consisting of 242 and 247 bp. The upper and lower bands in the polyacrylamide gel are 242 and 247 bp, respectively. Heterozygosity for DXS15-134 demonstrated both bands while homozygosity evidenced only the upper or lower band.

in studies with a large number of Japanese females. We have examined the heterozygosity of HUMARA in 110 Japanese females. To detect hematopoietic clonality from females without heterozygosity for HUMARA, we used the polymorphic STR site between DXS15 and DXS134 (DXS15-134) on Xq28 [10]. We report here the efficacy of clonality analysis for hematopoietic cells using the combined PCR of STR sites at HUMARA and DXS15-134.

MATERIALS AND METHODS

Blood and Bone Marrow Samples

Blood samples were obtained from 110 Japanese females after informed consent was given. Bone marrow was obtained from a patient with erythroleukemia (FAB subtype: M6) after informed consent was given. Mononuclear and polymorphonuclear cells were collected from blood and bone marrow using Ficoll-Paque (ρ = 1.077: Pharmacia Biotech, Sweden). Mononuclear cells of the bone marrow were separated into CD2+, CD14+, CD19+, and Glycophorin-A+ fractions by flow cytometry (FACStar^{PLUS}: Becton-Dickinson, San Jose, CA). The fraction of CD15+ cells was separated from polymorphonuclear cells. DNA was extracted from blood mononuclear cells and cell fractions from marrow by standard procedures.

PCR

DNA samples were divided into two aliquots. One was digested with *Hpa*II (Takara, Tokyo, Japan) while the other was incubated with digestion buffer alone. All reactions were performed at a total volume of 30 μl for 12 hr at 37°C. After digestion, reactions were terminated by incubating the mixture at 95°C for 10 min. From this reaction, 2 μl were added to a 30 μl total volume PCR reaction containing both oligonucleotide primers at a concentration of 1 μM, 250 μM dNTPs, 0.025 U Takara Ex Taq polymerase/μl (Takara), 0.2 μl of ^{32}P dCTP/μl (3,000 Ci/mmol)(Amersham, Buckinghamshire, England), 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.01% (w/v) gelatin. Wehnert et al. [10] reported the STR polymorphism of $(GTTTT)_2$+ $(GCTTT)_2$+$(GTTTT)_5$ between DXS15 and DXS134, with heterozygosity of 41% among caucasian females. Since the site digested with *Hpa*II is located in the reported sense primer, we designed a new primer for the site extending 8 bp to the 5′-end. The sequences were: primer 1, 5′-MMGAATTCTTTGCCTAGACCGG-3′; and primer 2, 5′-TTGAGCCAGGAGAATCGCTTGAAC-3′. M consisted of any nucleotide among A, C, G, and T. The sequences of the primers for STR of HUMARA were from Allen et al. [9]: primer 1, 5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′; and primer 2, 5′-TCCAGAATCTGTTCCAGAGCGTGC-3′. PCR conditions for DXS15-134 were the same as for HUMARA. DNA with or without *Hpa*II digestion was amplified using a Perkin-Elmer (Norwalk, CT) thermocycler for 30 cycles (each comprising 30 sec at 95°C, 30 sec at 62°C, and 30 sec at 72°C), with an initial denaturation at 95°C for 5 min. Two microliters of the PCR product were mixed with 5 μl of sequence gel-loading buffer (98% deionized formamide, 10 mM EDTA [pH 8.0], 0.025% xylene cyanol FF, and 0.025% bromophenol blue). Of this mixture 1.5 μl was denatured at 80°C for 5 min, then loaded on a denaturing 6% 39:1 acrylamide/bis-acrylamide gel (8 M urea and 1 × TBE). Electrophoresis was performed at 800 V for 6 hr. The gel was dried and exposed to an intensifing screen at room temperature for 2 hr. Autoradiographs were analyzed by a Bioimaging analyzer (Fuji Photo Film, Tokyo, Japan).

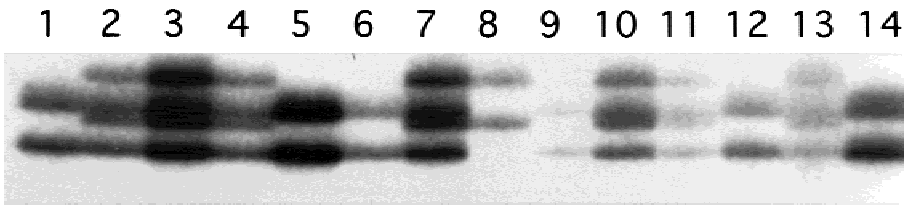

Fig. 2. Analysis of heterozygosity of STR at DXS15-134 in 14 normal females. PCR was performed on DNA samples without digestion by *Hpa*II to examine the heterozygosity of DXS15-134. Lanes 2,3,4,7,10,11,13: heterozygosity. Lanes 1,5,6,8, 9,12,14: homozygosity.

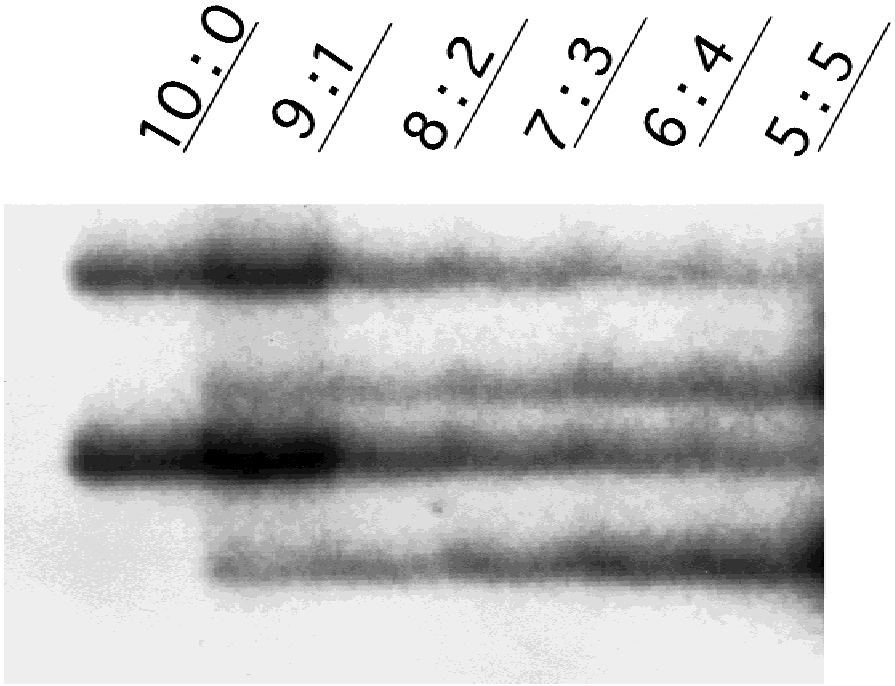

Fig. 3. Cell-mixing experiment using two males with different numbers of STR at DXS15-134. Cells were mixed in ratios shown at the upper part of the gel. PCR was performed on DNA samples without digestion by *Hpa*II for each of these mixtures.

RESULTS AND DISCUSSION

The polymorphic STR site between DXS15 and DXS134 (DXS15-134) is shown in Figure 1 (nucleotides 437 [HUMXREPB; GeneBank]; accession number L02680). The PCR products were 242 and 247 bp. To test for heterozygosity of DXS15-134, DNA was isolated from 110 Japanese females. PCR products electrophoresis formed four bands. Each allele was represented by two bands, which were designated as upper and lower bands (Fig. 1). Two bands formed from one allele because each single-stranded DNA migrates slightly differently in the gel due to complementary base composition. DNA from males was not amplified by the PCR method after *Hpa*II digestion. Among 110 females, 50 (46%) showed upper and lower bands, 15 showed only the upper band, and 35 showed only the lower band (Fig. 2). There is a possibility of allele-specific competition, where one allele may be more easily amplified than the other. However, there was no difference between the intensity of the upper and lower bands in DNA samples from females heterozygous for DXS15-134 (Fig. 2).

We examined the STR on HUMARA in 110 females and found heterozygosity in 74 of 110 (67%). When we combined the assays for DXS15-134 and HUMARA, the clonality of hematopoietic cells was detected in 91 of 110 females (83%); 36 samples with homozygosity for HUMARA showed 17 to be heterozygous (47%) and 19 to be homozygous (53%) for DXS15-134.

We examined the pattern of X-inactivation among 18 females who were heterozygous for both DXS15-134 and HUMARA loci. The results obtained for DXS15-134 correlated exactly with those of HUMARA. Of the 18 samples, 15 showed equal X-inactivation and 3 showed skewed X-inactivation (data not shown).

To demonstrate the quantitative potential of the PCR assay for DXS15-134, DNA from two males who had different bands at DXS15-134 were mixed and amplified. In Figure 3, a total of 0.2 μg of DNA was mixed at various ratios. The lower bands could be detected in a 9:1 mixture, indicating that the minor clone (10%) can be detected from blood samples.

We examined the clonality of each cell fraction separated by flow cytometry in a patient with erythroleukemia (FAB; M6) who was in clinical remission but with morphologically dysplastic marrow cells. PCR was performed on CD15+, CD2+, CD19+, glycophorin-A+, and

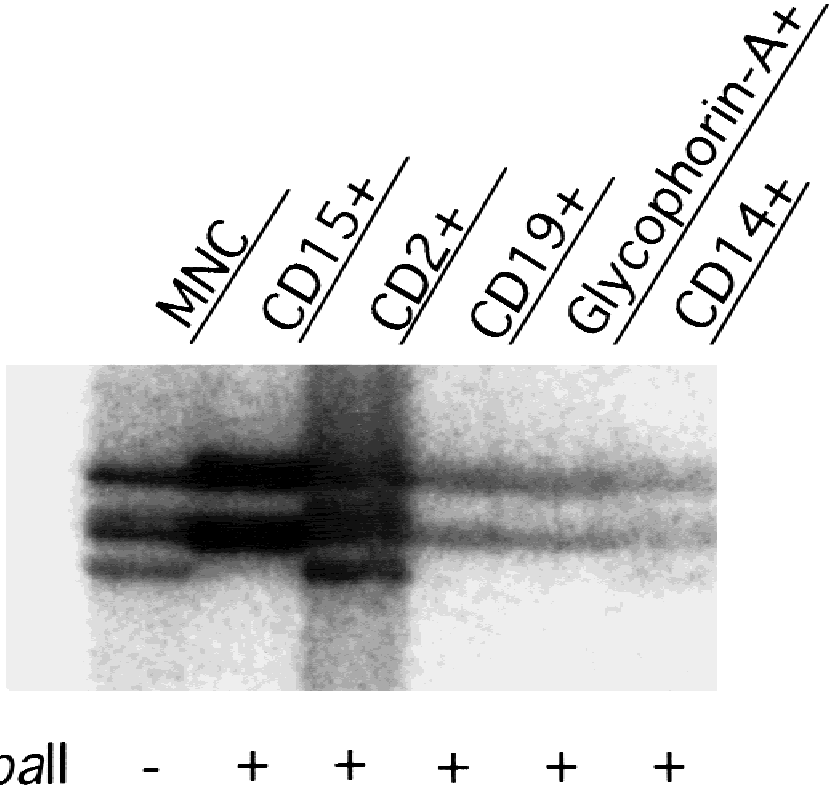

Fig. 4. Analysis of the clonality of each cell fraction separated by flow cytometry in a patient with erythroleukemia (M6) using the PCR method of DXS15-134. PCR was performed on DNA samples with digestion by *Hpa*II from each cell fraction and DNA without digestion by *Hpa*II from mononuclear cells (MNC). Each cell fraction is shown in the upper part of the gel. Cell fractions of CD15+, CD2+, CD19+, Glycophorin-A+, and CD14+ correspond to granulocyte, T lymphocyte, B lymphocyte, erythroblast, and monocytes, respectively.

CD14+ cell fractions. In hematologic disorders, T lymphocytes provide a reasonable control, because they are derived from primitive hematopoietic stem cells and have similar population dynamics as other blood cells in comparison with other tissue cells, such as skin [11]. Both upper and lower allele bands were observed in undigested mononuclear cells and CD2+ fraction, while only the upper allele was observed in CD15+, CD19+, glycophorin-A+, and CD14+ cell fractions (Fig. 4). This result indicates that the leukemic clone involved not only the myeloid lineage but also B lymphocytes despite a clinical remission. In fact, the patient relaped soon after.

The assay of this novel DXS15-134 site detects the clonality of blood cells in 85% of Japanese females when combined with an assay for HUMARA. Since the PCR conditions for DXS15-134 are the same as HUMARA, both procedures can be simultaneously performed in one clonality assay. This assay may be useful in the clonal analysis of very small numbers of blood cells.

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